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Deficient Proliferation of Myeloid, Erythroid, and Multipotent Progenitor Cells in Long-Term Marrow Cultures From Patients With Aplastic Anemia Treated With Immunosuppressive Therapy

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By using Dexter-type long-term marrow cultures (D-LTMC), it has been shown previously that hematopoietic progenitor cells (HPC) from patients with aplastic anemia (AA) have a deficient proliferation in vitro. The studies reported to date, however, have focused exclusively on granulomonocytic progenitors and no information exists on erythroid or multipotent progenitor cells. On the other hand, in such studies, the input progenitor cell numbers were significantly below normal levels, thus suggesting that the rapid disappearance of myeloid progenitor cells from AA D-LTMC could also be due, at least in part, to their reduced number at culture onset. In the present study, we have followed the kinetics of myeloid, erythroid, and multipotent progenitors, from 24 AA patients subjected to immunosuppressive therapy (including patients that achieved complete, partial, or no remission at all), throughout a seven-week culture period. For analysis, we grouped all the patients based on their initial content of all three types of progenitors. Thus, we were able to evaluate separately the kinetics of these cells in D-LTMC from patients with normal and subnormal levels of progenitor cells. At the time of marrow sampling, most patients showed decreased levels of HPC; in fact, only 21%, 8%, and 16% of them showed normal levels of myeloid, erythroid, and multipotent progenitors, respectively. When cultured in D-LTMC, HPC from all AA patients analyzed showed a relatively fast disappearance from the cultures. Indeed, myeloid progenitors could be detected for only six weeks, whereas erythroid and multipotent progenitors disappeared from the cultures after two and one weeks of culture, respectively. In contrast, in normal marrow D-LTMC, myeloid, erythroid, and multipotent progenitors were detected for at least seven, five, and three weeks, respectively. Such a deficient proliferation was observed even in cultures of AA patients that contained normal levels of HPC at culture onset. Interestingly, no correlation was found between HPC proliferation in D-LTMC and response to treatment. Thus, the results of this study indicate the presence of a functional in vitro deficiency in the hematopoietic system of patients with AA, including those that achieved partial or complete remission after immunosuppressive treatment. Furthermore, this work suggests that such a proliferation deficiency is more pronounced in erythroid and multipotent progenitors than in their myeloid counterparts. Am. J. Hematol. 59:149–155, 1998.

Key words: aplastic anemia; bone marrow; hematopoiesis; long-term marrow cultures; progenitor cells

INTRODUCTION

Aplastic anemia (AA) is a clear example of bone marrow failure. This disease results from the inability of hematopoietic stem/progenitor cells (HSPC) to produce adequate numbers of erythrocytes, leukocytes, and plate-

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lets, so that patients show pancytopenia and a hypocellular bone marrow [1,2]. Several studies have shown decreased levels of both $CD34^+$ and colony-forming cells in the marrow and peripheral blood of AA patients [3,4]. Moreover, the levels of functionally primitive hematopoietic cells, including long-term culture initiating cells (LTC-IC), as well as cobblestone area-forming cells (CAFC), are also reduced [5,6]. Interestingly, in a major proportion of patients, immunosuppressive treatment significantly increases HSPC numbers; however, such numbers usually remain below normal levels [4,5].

Although it is clear that HSPC numbers are dramatically reduced in AA, the functional integrity of these cells, particularly those from patients in partial or complete remission, is still under investigation. Dexter-type long-term marrow culture (D-LTMC) constitutes one of the best in vitro systems for the assessment of the proliferation potential of HSPC and to study their interaction with the hematopoietic microenvironment (HM) [7,8]. Previous studies by Gibson and Gordon-Smith [9] and by Marsh et al. [10] have shown deficient growth of granulomonocytic progenitor cells in D-LTMC of AA patients, as compared with normal marrow D-LTMC, thus suggesting functional alterations in AA myeloid progenitors. To date, however, the growth of AA-derived erythroid and multipotent progenitor cells in this experimental system has not been reported. On the other hand, in both of the above studies, the input progenitor cell number in AA D-LTMC was significantly lower than in normal marrow cultures, indicating that the faster disappearance of myeloid progenitor cells from AA cultures could also be due, at least in part, to their reduced number at culture onset.

In the present study, we have addressed these issues by assessing the long-term in vitro growth of myeloid, erythroid, and multipotent progenitor cells from 24 patients with AA subjected to immunosuppressive treatment. For analysis, we grouped the cultures based on their initial content of all three types of progenitors; thus, we were able to evaluate separately the kinetics of these cells in D-LTMC from patients with normal and subnormal levels of progenitor cells. Furthermore, in trying to define in more detail the possible correlation between clinical and laboratory findings, we compared progenitor cell numbers and long-term growth in D-LTMC between patients that responded to treatment and those who did not.

MATERIALS AND METHODS

Cell Collection

Bone marrow (BM) cells, collected according to institutional guidelines, were obtained from 24 patients with AA. Diagnosis was established by BM biopsy and peripheral blood count criteria according to the International Agranulocytosis and Aplastic Anemia Study group [11]. By the time of this study, all the patients had been subjected to immunosuppressive treatments. Information on patients is shown in Tables I and II. BM samples were also obtained from the iliac crest of eight bone marrow transplant donors at the ''Bernardo Sepulveda'' Hospital, National Medical Center, Mexico City. The ethical committee of the National Medical Center has approved these procedures.

TABLE I. Clinical Data on the 24 AA Patients Included in This Study*

PN	Sex	Age (years)	Time from Dx (years)	Treatment	Response	Transfusion requirement
1	M	54	15	ALG, CSA, GM	CR	No
2	M	20	3	ALG, CSA, G	PR	No
3	F	28	<1	ALG, CSA, GM	PR	No
4	M	24	2	ALG, CSA, GM	NR	Yes
5	F	23	11	CSA	PR	No
6	M	56	1	CSA	NR	Yes
7	F	19	<1	ALG, CSA, G	PR	No
8	M	22	2	ALG, CSA, G	NR	Yes
9	M	52	3	CSA	NR	Yes
10	F	48	4	LC	CR	No
11	M	28	<1	CSA	NR	Yes
12	M	46	25	LC	PR	No
13	F	54	2	CSA, GM	PR	Yes
14	M	24	<1	ALG, CSA, G	PR	No
15	M	55	2	ALG	CR	No
16	F	32	2	CSA	PR	No
17	F	55	3	ALG	MDS	No
18	M	38	3	ALG, CSA, GM	CUE	No
19	M	22	3	ALG	CR	No
20	F	56	4	LC	PR	No
21	F	52	3	LC	CR	No
22	M	38	1	ALG, CSA, G	CUE	Yes
23	M	23	1	LC	PR	No
24	F	52	3	LC, CSA	PR	No

*AA, aplastic anemia; PN, patient number; Dx, diagnosis; ALG, antilymphocyte globulin; CSA, cyclosporine A; GM, GM-CSF; G, G-CSF; LC, lymphocytapheresis; CR, complete response; PR, partial response; NR, no response; MDS, evolution to myelodysplatic syndrome; CUE, currently under evaluation.

All patients but number 5 were diagnosed as having severe AA. Patient 5 was diagnosed as having nonsevere AA.

Cell Processing

Buffy coat cells, both from AA and normal BM, were obtained by centrifugation (400*g* for seven min) and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2% fetal bovine serum (FBS) (StemCell Technologies Inc. [STI], Vancouver, BC, Canada). Total numbers of nucleated and viable cells were determined with a hemocytometer, using Turck's solution and trypan blue stain, respectively.

Hematopoietic Colony Assays

In two patients before treatment and in all 24 patients after treatment, hematopoietic progenitor cells were as-

TABLE II. Hematological Data on the 24 Patients Included in This Study*

PN	Hemoglobin (g/dl)	Hematocrit (%)	Leukocytes ($\times 10^9$/l) [% neutrophils]	Platelets ($\times 10^9$/l)
1	8.0	24	3.2 [40]	18,000
2	7.1	20	2.5 [30]	17,000
3	7.5	22	1.8 [35]	11,000
4	7.3	21	2.3 [55]	7,000
5	9.0	27	3.6 [60]	22,000
6	7.0	21	2.5 [50]	8,000
7	6.8	18	1.5 [30]	15,000
8	7.0	21	1.2 [30]	12,000
9	6.0	17	5.7 [40]	13,000
10	9.5	27	3.7 [55]	32,000
11	7.0	21	2.2 [30]	21,000
12	5.0	15	1.8 [30]	18,000
13	9.0	27	3.3 [40]	21,000
14	7.5	23	2.7 [30]	22,000
15	13.3	42	4.0 [70]	62,000
16	9.0	27	3.5 [45]	32,000
17	9.7	29	3.3 [40]	35,000
18	11.3	33	6.1 [67]	18,000
19	16.0	45	8.5 [70]	235,000
20	10.5	30	3.3 [65]	32,000
21	13.1	39	3.7 [60]	34,000
22	6.0	18	1.5 [30]	12,000
23	8.6	24	2.7 [67]	24,000
24	9.0	27	3.3 [50]	20,000

*Hematological data at the time of this study. PN, patient number.

sayed in methylcellulose-based semisolid cultures (STI, Vancouver, Canada). The culture medium consisted of 0.9% methylcellulose, 30% FBS, 1% bovine serum albumin, 10^{-4} M 2-mercaptoethanol, two mM L-glutamine, 50 ng/ml recombinant human (rh) stem cell factor (SCF), 10 ng/ml rh interleukin-3 (IL-3), 10 ng/ml rh granulocyte-macrophage colony-stimulating factor (GM-CSF) and three U/ml rh erythropoietin (EPO). Buffy coat cells, both from AA and normal BM, were plated at a final concentration of 5×10^4 cells/ml and the cultures were incubated at 37°C in an atmosphere of 5% CO_2 in air. Nonadherent cells from D-LTMC were also cultured in this manner; however, the plating cell concentration varied from 5×10^4 to 1×10^4, depending on the cell number recovered from the cultures. After 14–17 days of culture, colonies were scored in the same dish using an inverted microscope. Hematopoietic colonies were classified as described previously [12]: CFU-MIX, colonies containing both erythroid and myeloid cells; CFU-E, erythroid clusters of 20–50 hemoglobinized cells; BFU-E, erythroid colonies of more than 50 hemoglobinized cells grouped in one or several clusters. Myeloid colonies comprised the identifiable subpopulations of pure granulocytic colonies (CFU-G), pure macrophagic colonies (CFU-M), and colonies containing both granulocytes and macrophages (CFU-GM).

Long-Term Marrow Cultures

D-LTMC were established as described previously [13]. Buffy coat cells were resuspended in LTMC medium (STI, Vancouver, Canada) at a final concentration of 3×10^6 cells per ml. The LTMC medium composition is as follows: Alpha medium supplemented with 12.5% horse serum, 12.5% FBS, 0.2 mM inositol, 20 μM folic acid, 10^{-4} M 2-mercaptoethanol, 2 mM L-glutamine, and freshly dissolved hydrocortisone to yield a final concentration of 10^{-6} M. The cell suspension was loaded into 24-well plates (one ml/well) and incubated at 37°C in an atmosphere of 5% CO_2 in air. After three days, cultures were transferred to a different incubator and maintained at 33°C. Four days later (seven days after initiation of the culture), half of the supernatant and nonadherent cells were removed from the wells and replaced with fresh culture medium. The cultures were processed in this manner at weekly intervals. The nonadherent cells, obtained weekly during the medium change, were counted, morphologically analyzed, and assayed for hematopoietic progenitors.

Statistics

Statistical analysis was performed by using the Mann-Whitney U test.

RESULTS

Hematopoietic Progenitor Cell Content

In normal bone marrow colony assays, the median number for myeloid progenitors was 238 (range 129–422) per 10^5 nucleated cells. Similar numbers were observed for erythroid progenitors (median 194; range 106–285), whereas multipotent progenitors showed a median of six (range 2–35) per 10^5 nucleated cells.

Progenitor cell numbers in patients with AA showed great variability (Fig. 1). Twenty-one percent of the patients (five out of 24; patients 6, 7, 16, 18, and 19) showed myeloid progenitor numbers within the normal range. Only 8% of the patients (two out of 24; patients 7 and 19) showed normal levels of erythroid progenitors, whereas in 16% of the patients (four out of 24; patients 8, 16, 17, and 19) the numbers of multipotent progenitors were within the normal range. Patient 19 was the only one with normal levels for all three types of hematopoietic progenitors.

Forty-six percent of the patients (11 out of 24; patients 1, 2, 3, 8, 10, 11, 12, 13, 14, 17, and 20) showed reduced numbers of both myeloid and erythroid progenitors. Sixteen percent of the patients (four out of 24; patients 4, 9, 15, and 21) showed reduced myeloid progenitor cell growth with total absence of erythroid progenitors. In contrast, no cultures were observed in which erythroid progenitors could be found with total absence of myeloid

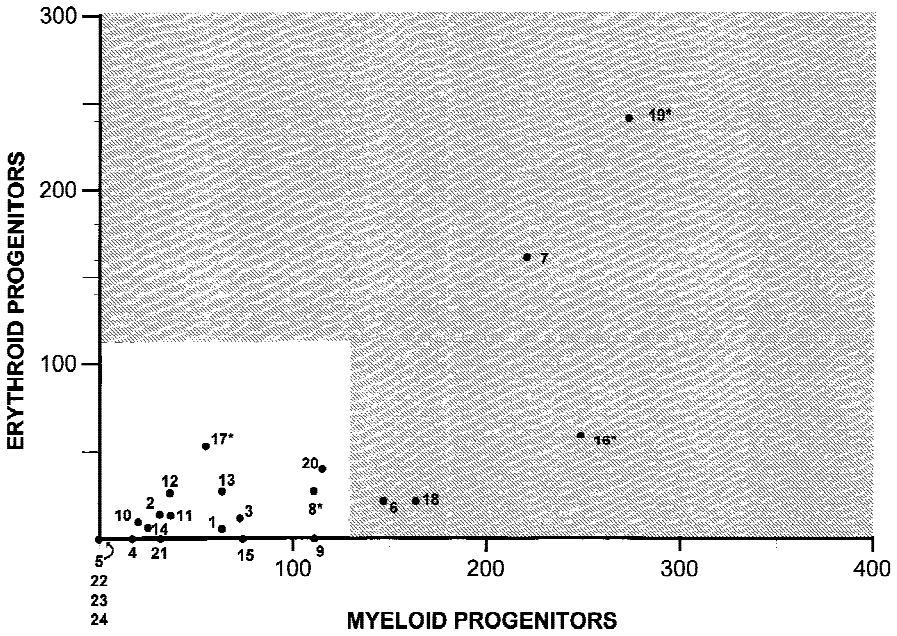

Fig. 1. Colony-forming cell (CFC) content in the 24 patients with AA included in this study. The results shown (CFC/10^5 nucleated cells) were obtained at the time of study (i.e., after immunosuppressive therapy). Only four patients (*) showed CFU-MIX growth: patients 8, 16, and 17 each contained two CFU-MIX/10^5 nucleated cells; patient 19 contained 16 CFU-MIX/10^5 nucleated cells. In two of the patients (patients 3 and 7), CFC content was determined at diagnosis (before) and after treatment. In cultures of patient 3 before treatment, no colonies were observed. In cultures of patient 7 before therapy, only 12 myeloid colonies were observed. Normal range for myeloid (CFU-G + CFU-M + CFU-GM) progenitors: 129–422/10^5 nucleated cells; normal range for erythroid (CFU-E + BFU-E) progenitors: 106–285/10^5 nucleated cells; normal range for multipotent progenitors: 2–35/10^5 nucleated cells. Normal levels of myeloid and erythroid progenitors are indicated by the shaded area.

progenitors. No patients were observed with CFU-MIX levels >0 but below the normal range. Finally, 16% of the patients (four out of 24; patients 5, 22, 23, and 24) showed no hematopoietic colony growth at all (Fig. 1). It is noteworthy that in two of the patients (patients 3 and 7), colony assays were performed both at the time of diagnosis and after immunosuppressive therapy. At the time of diagnosis, patient 3 showed no colony growth at all, whereas in the semisolid culture of patient 7, only 12 myeloid colonies were observed (not shown). Thus, these results show that in the two patients evaluated before and after treatment, a clear increase in progenitor cell number occurred after immunosuppressive therapy.

Based on the growth of hematopoietic progenitors in semisolid cultures, five groups were identified: Group 1 comprised patients with normal levels of myeloid progenitors (median 224 [144–274] per 10^5 nucleated cells; patients 6, 7, 16, 18, and 19). Group 2 included patients with reduced levels of myeloid progenitors (median 56 [14–120] per 10^5 nucleated cells; patients 1, 2, 3, 4, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, and 21). Group 3 comprised patients with normal levels of erythroid progenitors (median 204 [168–240] per 10^5 nucleated cells; patients 7 and 19). Group 4 included patients with reduced levels of erythroid progenitors (median 21 [10–66] per 10^5 nucleated cells; patients 1, 2, 3, 6, 8, 10, 11, 12, 13, 14, 16, 17, 18, and 20). Finally, group 5 comprised patients with normal levels of multipotent progenitors (median 2 [2–16] per 10^5 nucleated cells; patients 8, 16, 17, and 19).

Nucleated Cell Kinetics in D-LTMC

Hematopoietic cell kinetics in D-LTMC was analyzed according to the group distribution mentioned above. In terms of total nonadherent cells, the numbers observed in cultures of group 1 during the first three weeks were very similar to those in normal BM cultures (Table III). However, from week 4, a drastic drop was observed in the former cultures, so that, between weeks 4 and 7, the median cell number in normal marrow cultures was fourfold higher (Table III). In cultures of group 2, only during the first week of culture, the total number of nonadherent cells was similar to those in cultures of normal bone marrow; afterwards, their levels were always significantly lower (3- to 13-fold). It is noteworthy that, from week 4, the total numbers of nonadherent cells in cultures of AA groups 1 and 2 were similar (Table III).

Progenitor Cell Kinetics in D-LTMC

Progenitor cell kinetics in D-LTMC was also analyzed according to the group distribution mentioned above. As shown in Table IV, during the first three weeks of culture, the absolute numbers of myeloid progenitors in cultures of group 1 were within the range observed in normal marrow cultures. By week 4, however, a pronounced drop in myeloid progenitor cell levels was observed in group 1 cultures and from week 5, these progenitors could not be detected in cultures of four of the five patients included in this group. As expected, median numbers of myeloid progenitors in cultures of group 2 were

TABLE III. Total Number of Nonadherent Cells in D-LTMC From Normal and AA Marrow†

Week	Normal	AA group 1	AA group 2
0	3.00	3.00	3.00
1	1.43 (1.12–2.57)	1.07 (0.50–2.00)	0.95 (0.30–2.90)
2	0.80 (0.36–1.92)	1.04 (0.92–1.57)	0.28 (0.16–0.78)*
3	0.37 (0.08–0.99)	0.41 (0.10–0.50)	0.11 (0.06–0.34)*
4	0.21 (0.04–0.73)	0.05 (0.03–0.14)*	0.07 (0.01–0.29)*
5	0.10 (0.03–0.24)	0.03 (0.01–0.06)*	0.02 (0–0.13)*
6	0.05 (0.02–0.13)	0.01 (0.01–0.03)*	0.01 (0–0.03)*
7	0.04 (0.01–0.10)	0.01 (0–0.02)*	0.003 (0–0.009)*

†Results (nucleated cells × 10^{-6}/well) correspond to median and range. Normal: n = 8. Group 1: Patients 6, 7, 16, 18, 19. Group 2: Patients 1, 2, 3, 4, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 21. D-LTMC, Dexter-type long-term marrow cultures; AA, aplastic anemia.
*Significantly different ($P < 0.05$) from normal.

TABLE IV. Total Number of Myeloid Progenitors in D-LTMC From Normal and AA Marrow†

Week	Normal	AA group 1	AA group 2
0	7,140 (3,870–12,660)	6,660 (4,320–8,220)	1,680 (420–3,600)*
1	3,643 (1,387–10,134)	1,780 (840–4,680)	176 (21–4,413)*
2	872 (444–6,682)	924 (558–2,496)	32 (0–2,488)*
3	547 (72–3,288)	310 (20–1,009)	9 (0–285)*
4	496 (41–2,281)	26 (0–360)*	0 (0–58)*
5	119 (18–932)	0 (0–67)*	0 (0–16)*
6	34 (10–108)	0 (0–12)*	0 (0–4)*
7	36 (7–138)	0*	0*

†Results (myeloid progenitors/well) correspond to median and range. Myeloid progenitors = CFU-G + CFU-M + CFU-GM. Normal: n = 8. Group 1: Patients 6, 7, 16, 18, 19. Group 2: Patients 1, 2, 3, 4, 8, 9, 10, 11, 12, 13, 14, 15, 17, 20, 21. D-LTMC, Dexter-type long-term marrow cultures; AA, aplastic anemia.
*Significantly different ($P < 0.05$) from normal.

always significantly lower than in normal marrow D-LTMC (Table IV). Just like for total nonadherent cells, no difference was observed between myeloid progenitor levels in groups 1 and 2, after four weeks of culture.

In terms of erythroid progenitors, an early drop in their numbers was observed both in groups 3 and 4 (Table V). In fact, from week 2 of culture, practically no erythroid progenitors could be detected in D-LTMC from AA patients. This was particularly surprising for cultures of group 3, which, on day 0, showed slightly higher levels of erythroid progenitors than normal marrow cultures. Finally, an even earlier disappearance was observed for multipotent progenitors in cultures of group 5. Indeed, after only one week of culture, their median number was zero (Table VI). This was in clear contrast to what we observed in normal marrow cultures, in which multipotent progenitor cells were present for, at least, the first three weeks of culture.

DISCUSSION

One of the most salient features of patients with AA is their reduced content of HPC in bone marrow. Although

TABLE V. Total Number of Erythroid Progenitors in D-LTMC From Normal and AA Marrow†

Week	Normal	AA group 3	AA group 4
0	5,820 (3,180–8,560)	6,120 (5,040–7,200)	630 (300–1,620)*
1	2,461 (452–7,084)	1,447 (480–2,414)	41 (0–392)*
2	230 (26–744)	0*	0 (0–4)*
3	30 (4–706)	0*	0*
4	19 (0–161)	0*	0*
5	4 (0–114)	0*	0*

†Results (erythroid progenitors/well) correspond to median and range. Erythroid progenitors = CFU-E + BFU-E. Normal: n = 8. Group 3: Patients 7, 19. Group 4: Patients 1, 2, 3, 6, 8, 10, 11, 12, 13, 14, 16, 17, 18, 20. D-LTMC, Dexter-type long-term marrow cultures; AA, aplastic anemia.
*Significantly different ($P < 0.05$) from normal.

TABLE VI. Total Number of Multipotent Progenitors in D-LTMC From Normal and AA Marrow†

Week	Normal	AA group 5
0	180 (30–1050)	60 (60–480)
1	165 (1–805)	0 (0–20)*
2	40 (0–153)	0*
3	5 (0–42)	0*

†Results (multipotent progenitors/well) correspond to median and range. Normal: n = 8. Group 5 = Patients 8, 16, 17, 19. D-LTMC, Dexter-type long-term marrow cultures; AA, aplastic anemia.
*Significantly different ($P < 0.05$) from normal.

it has been clearly established that immunosuppressive therapy significantly increases HPC levels in most of these patients, the functional integrity of such cells is still under investigation. In the present study, we have determined the hematopoietic progenitor cell content in bone marrow of 24 AA patients after immunosuppressive treatment and characterized the long-term proliferation of such cells in D-LTMC.

In two of the patients (patients 3 and 7) colony assays were performed both at the time of diagnosis and after immunosuppressive therapy. At the time of diagnosis, patient 3 showed no colony growth at all, whereas in the semisolid culture of patient 7, only 12 myeloid colonies were observed. After treatment, significant increments in myeloid and erythroid colony-forming cell numbers were achieved in both patients. Multipotent progenitor cells, however, could not be detected neither before nor after treatment. Similar results were reported by Schrezenmeier et al. [6], who found significant increments in CFU-GM, BFU-E, and CFU-E levels in treated patients and saw no significant difference in the numbers of more primitive (CAFC) progenitors before and after treatment. The relevance of this latter observation, however, is still unclear; indeed, others have found significant recoveries in LTC-IC levels after treatment [5], thus indicating that immunosuppression has an effect at the level of primitive progenitor cells.

Semisolid cultures from the 24 treated patients showed

great variability in colony-forming cell numbers and lower than normal levels of such progenitors in most patients. Indeed, only 21%, 8%, and 16% of the patients showed normal levels of myeloid, erythroid, and multipotent progenitors, respectively. These results are in agreement with previous studies showing reduced numbers of primitive and mature progenitor cells in treated patients, as compared with normal subjects [5,6]. Interestingly, no correlation was found between progenitor cell content after therapy and response to treatment. In fact, only one of the five patients that achieved complete remission showed normal levels of hematopoietic progenitors. In contrast, one of the patients that was refractory to treatment had normal numbers of myeloid progenitors. Moreover, patients 5, 23, and 24, who achieved partial remission, showed no colony growth at all.

In the two patients analyzed before and after therapy, we could not find any correlation between their in vivo response to treatment and the increments in colony-forming cell numbers observed. Both of them achieved partial remission in vivo; in fact, their hematological parameters at time of this study were very similar (Table 2). However, whereas patient 3 showed a modest, although significant, increment in progenitor cell numbers (Fig. 1), patient 7 reached normal levels for both myeloid and erythroid progenitors (Fig. 1). In their study, Maciejewski and colleagues [5] found no correlation between LTC-IC content at the time of presentation and response to treatment. Thus, taking all of the above data together, it seems that in vitro progenitor cell growth in AA is not a reliable predictor test for patient's outcome.

D-LTMC constitutes a suitable experimental system for the assessment of the in vitro proliferation capacity of HSPC [7,8]. Previous studies have shown deficient hematopoiesis (i.e., rapid disappearance of HPC from culture) in D-LTMC of AA patients, as compared with normal marrow D-LTMC [9,10]. Those studies, however, focused exclusively on myeloid progenitors, and no data was presented on erythroid or multipotent cells. Furthermore, in both of these studies, the input progenitor cell number was significantly lower than in normal marrow cultures, thus suggesting that the faster disappearance of progenitor cells from the cultures could be due, at least in part, to their reduced number at culture onset.

In the present study, we have followed the proliferation kinetics of myeloid, erythroid, and multipotent progenitors in AA D-LTMC. For analysis, we grouped these patients based on their initial content of all three types of progenitors, thus we were able to separately evaluate patients with normal and subnormal levels of these cells at culture onset. Our results show that in all groups of patients, including those with normal levels of HPC, the long-term proliferation of these cells was clearly deficient, as compared with normal marrow D-LTMC. Such a deficiency was more evident for erythroid and multipotent progenitor cell proliferation. Although the nature of this deficiency is still unclear, some insights into this issue have been obtained. Indeed, several groups have suggested that there are intrinsic functional abnormalities in AA progenitor cells, including LTC-IC, that could be involved in: 1. their reduced capacity to produce secondary colonies in vitro; 2. their deficient ability to grow in the absence of factors produced by accessory cells; and 3. their increased sensitivity to undergo apoptosis [4–6,14,15]. To date, however, it is not known whether these functional abnormalities involve the whole stem cell compartment or only a subset of such cells.

Among all 24 patients, patient 19 was the only one with normal levels of myeloid, erythroid, and multipotent progenitors at the time of marrow sampling. This correlated with the fact that his hematological parameters were completely normal and that he has been in complete remission for the last three years. When plated in D-LTMC, his myeloid progenitor cells showed proliferation kinetics similar to that observed in normal cultures. In contrast, erythroid and multipotent progenitors showed deficient proliferation, as compared with normal marrow progenitors. The actual reason for this observation is not clear. One possible explanation is that erythroid and multipotent progenitors in this patient have been selectively affected and, thus, are unable to develop in D-LTMC. A second possibility could be that the HM developed in vitro is abnormal, in terms of its composition and/or function, thus, it is unable to provide some factor(s) required mostly by erythroid and multipotent cells. Further studies with bone marrow cells from this patient will be required to address these points.

Regarding the points mentioned above, it is noteworthy that although we observed that erythropoiesis seemed to be more affected than myelopoiesis (i.e., in almost all patients erythroid progenitor cells are reduced to a larger extent than are myeloid progenitors and the former show a more deficient proliferation in LTMC than the latter), no correlation seems to exist with the hematological status of the patients. That is to say, we could not conclude that patients were more anemic than neutropenic. Moreover, we did not find any correlation between hematocrit and hemoglobin levels in circulation and erythroid progenitors levels in bone marrow. Also, we could not find any correlation between leukocytes levels in circulation and myeloid progenitor cells in bone marrow.

The 24 patients included in this study constitute a heterogeneous group in terms of time from diagnosis, type of therapy, and response to therapy. Despite this, two major conclusions can be drawn: 1. HPC levels are subnormal in most AA patients after any type of immunosuppressive therapy; and 2. regardless of the immunosuppressive treatment, HPC from AA patients show deficient long-term proliferation in vitro. This was observed even in cultures of patients that did respond to

treatment and that were in complete remission at the time of the study. These results indicate that there is no correlation between the clinical parameters of the patients, including type of treatment and outcome, and the in vitro functionality of their hematopoietic system. Our results, however, do not allow us to distinguish between functional abnormalities of the progenitor cells, as suggested by several groups [3–6,16,17], and functional abnormalities of the HM, as suggested by others [18,19]. Furthermore, we cannot rule out the possibility that, in some cases, abnormal lymphocytes, producing inhibitory cytokines, may still be present in culture, thus inhibiting the growth of HPC. Studies are in progress to address these possibilities.

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